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Mechanisms of C_2-ceramide-induced apoptosis in osteoblasts

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ABSTRACT

Dysregulated osteoblast programmed cell death (PCD) is implicated in metabolic bone diseases such as osteoporosis. Ceramide, a precursor to all complex sphingolipids, is a secondary messenger in PCD. However, the mechanisms of ceramide induced-PCD are poorly characterized. Thus, the aim of this study was to investigate the nature and mechanism of ceramide-induced PCD in osteoblasts. The MTT assay, propidium iodide uptake with flow cytometric analysis, and TUNEL staining were used to assess the effect of exogenous ceramide on cell viability and apoptosis in MC3T3-E1 cells and primary murine osteoblasts. Western blotting and immunofluorescence approaches were used to elucidate the underlying signaling mechanisms. Ceramide reduced MC3T3-E1 and primary osteoblast viability and induced apoptosis in a dose-dependent manner. Ceramide failed to activate the executioner caspases-3 and -7 and poly (ADP-ribose) polymerase (PARP), while a selective inhibitor of caspase-8 abrogated ceramide effects on osteoblast viability. Ceramide induced p38 and ERK activation, but only p38 was involved in ceramide-induced osteoblast PCD. Our study highlights a novel role for ceramide in inducing caspase-8 and p38-dependent osteoblast PCD. Understanding cell death mechanisms in osteoblasts could help in the development of new targeted therapeutics that selectively identify cells with pathogenic programmed cell death pathways.

Keywords: Apoptosis, cell biology, osteoblasts

1. INTRODUCTION

Systemic and local mediators can dysregulate bone remodeling and contribute to the pathogenesis of diseases such as osteoarthritis and osteoporosis. Most metabolic disorders result from an imbalance between bone resorption and deposition by osteoclasts and osteoblasts, respectively (Zaidi, 2007). The maintenance of adult skeletal mass is not only controlled by changes in osteoclast and osteoblast function, but also their lifespan (Marie, 2015). The average normal osteoclast and osteoblast lifespan is 1-25 days and 1-200 days,

respectively, with cell life span regulated by programmed cell death (PCD) (Manolagas and Parfitt, 2010).

Cell death can result from three main pathways: apoptosis, autophagy, and necrosis (Kroemer et al., 2009), which can be distinguished by their cellular and morphological differences. The hallmarks of apoptosis are cytoskeletal breakdown, chromatin condensation, caspase-dependent DNA fragmentation, plasma membrane blebbing, cell shrinkage, and finally the shedding of apoptotic bodies (Kroemer et al., 2009). The morphological and biochemical changes of apoptosis are controlled by a cysteine proteases family known as caspases (Nicholson and Thornberry, 1997; Strasser et al., 2000; Thornberry et al., 1997). By contrast, autophagic cell death is characterized by cytoplasm and organelle degradation in double- or multi-membrane autophagy vesicles, which is usually a caspase-independent process (Gozuacik and Kimchi, 2004; Pattingre et al., 2009). In necrosis, cell swelling, plasma membrane rupture, and cell lysis occur (Kroemer et al., 2009).

Ceramide, a precursor of all complex sphingolipids, with sphingosine-1-phospate (S1P) constitutes a "sphingolipid" rheostat that determines cell fate. In many settings, ceramide is pro-apoptotic, and S1P mitigates this apoptotic effect (Hannun and Obeid, 2008; Spiegel and Milstien, 2003). In addition, ceramide-induced cell death often involves the stress-activated protein/mitogen-activated protein kinase (SAP/MAPK) pathways. The MAPKs are a superfamily of serine/threonine kinases that regulate diverse signaling transduction cascades including cellular proliferation, differentiation, migration, and death. In mammals, MAPKs include the extracellular signal-related kinases (ERKs), c-Jun-N-terminal kinase (JNK), and the p38 family of kinases (Chang and Karin, 2001; Turjanski et al., 2007). Ceramide has also been implicated in a several signaling cascades involving protein kinases such as protein kinase C zeta type (PKCz) (Bourbon et al., 2001), kinase suppressor of Ras (KSR) (Zhang et al., 1997), and protein phosphatases such as PP2A and PP1 (Ruvolo et al., 2002).

Unraveling the complexities of ceramide-mediated signaling in cell death is therefore of significant interest. Therefore, the aim of our study was to investigate the nature of osteoblast-induced apoptosis in response to ceramide and to elucidate the underlying mechanism.

2. MATERIALS AND METHODS

Cell culture

The murine osteoblastic cell line MC3T3-E1 (Sudo et al., 1983) and the transformed human osteoblastic cell line from a primary osteosarcoma, SaOS-2, were purchased from ATCC (Manassas, VA). Primary murine osteoblasts (POBs) were isolated from mice by sequential collagenase digestion of neonatal calvariae as previously described (Heath et al., 1984). Cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% batch-tested fetal bovine serum (FBS; Autogen Bioclear, Nottingham, UK), antibiotics, and L-glutamine (5 mM) (Sigma Aldrich, St Louis, MO). POB cultures were additionally supplemented with 50 µg/ml ascorbic acid. All cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

Reagents and antibodies

The following reagents were purchased from Sigma Aldrich: D-erythro-N acetylsphinganine (C_2-dihydroceramide, C_2-DHC), N-acetyl-D-sphingosine (C_2-ceramide), aurinitricarboxylic acid (ANCA), propidium iodide (PI), 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenlytetrazolium bromide (MTT), and Hoechst 33324. The following reagents were from Calbiochem (EMD Chemicals, San Diego, CA): cell-permeable pan-caspase inhibitor (DEVD-CHO), cell permeable caspase-8 inhibitor I (IETD-CHO), caspase-9 inhibitor I (Z-LE(OMe) HD (OMe)-FMK), a potent cell-permeable inhibitor of p38 MAPK (SB 202190), a selective cell-permeable inhibitor of MEK (PD 98059), and a potent cell-permeable inhibitor of protein kinases that induces apoptosis in human glioma cells (staurosporine; SS). Antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38 MAP kinase, anti-phospho-p38 MAPK kinase, anti-p44/p42, anti-phospho-p44/p42, anti-AIF, anti-PARP, anti-caspase 3, and anti-caspase 7 primary antibodies and goat anti-rabbit IgG HRP-linked secondary antibody. Alexa Fluor 555 goat anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA), and Vecta shield mounting medium with DAPI was purchased from Vector Laboratories Ltd. (Peterborough, UK).

Analysis of osteoblast viability

The MTT assay was used to measure cell viability as previously described (Hill et al., 1997). In brief, osteoblasts (5 x 10^3 cells/96-well) were treated with the reagents as indicated, and the solubilized formazan following the MTT reaction was measured on a microplate reader at 562-620 nm.

Analysis of osteoblast PCD (apoptosis)

PCD/apoptosis of osteoblasts was assessed and measured several different ways. In all experiments in this section, cells were plated at 1-2 x 10^4 cells/cm^2.

Assessing PI uptake by flow cytometry

Propidium iodide (PI) uptakewas used to measure the i*n vitro* loss of plasma membrane integrity, which occurs in apoptotic cells. Osteoblasts were treated with the indicated reagents, stained with 50 μg/ml PI, measured with the BD FACS Canto TM II flow cytometer (10^4 cells were captured per condition), and analyzed with BD FACSD iva software. Results were expressed as percentage PI uptake (mean ± SD of five replicates).

TUNEL assay

TUNEL staining was used to assess single DNA breaks as previously described (Hill et al., 1997). In brief, the *in situ* cell death detection kit from Roche Applied Science (Mannheim, Germany) was used according to the manufacturer's instructions. Osteoblasts were treated with the indicated reagents, fixed, permeabilized, and then labelled with TUNEL reagent. Only bright green nuclei were considered positiveas visualized by fluorescent microscopy with a FITC filter (emission 614 nm).

Hoechst 33324 nuclear staining

Nuclear morphological changes were assessed after nuclear staining with Hoechst 3332. Briefly, osteoblasts were plated on coverslips, fixed, and then incubated with 1 μg/ml Hoechst 33324 for 10 min. Only cells with clearly fragmented nuclei or condensed chromatin were considered apoptotic as visualized by fluorescence microscopy with a DAPI filter (emission 460 nm).
Analysis of osteoblast apoptosis mechanisms

Western blot analysis

The protein levels of activated MAPK members, various caspases, and downstream targets were assessed by western blot analysis (WB). Briefly, osteoblasts (2 x 10^5 cells/6-well) were treated with the reagents indicated, lysed with 4.8% SDS, 8% sucrose, and 2 M urea, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Millipore Corp., Burlington, MA). Membranes were incubated with primary antibodies overnight at 4°C at 1:250 (anti-caspase 3, anti-caspase 7and anti-PARP antibodies) or for 1 h at 1:1000 (anti-p38 MAP kinase, anti-phospho-p38 MAPK kinase, anti-p44/p42, anti-phospho-p44/p42, anti-SAPK/JNK, and anti-phospho-SAPK/JNK antibodies) or at 1:10,000 (anti-tubulin antibody). Then, membranes were incubated with secondary goat anti-rabbit IgG HRP-linked and donkey anti-mouse IgG HRP-linked antibodies for 1 h at room temperature at 1:1000. Enhanced chemiluminescence was used to visualize immunocomplexes according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech, Amersham, UK).

Caspase activity assay

The fluorochrome-labelled inhibitors of caspases (FLICA) assay were used to assess caspase involvement by measuring their activity. Briefly, the carboxyfluorescein FLICA apoptosis detection kit caspase assay from Immunochemistry Technologies (Oxford, UK) was used according to the manufacturer's protocol. Osteoblasts (1 x 10^5 cells/12-well) were treated with the indicated reagents, stained with FLICA reagent, measured with the BD FACS CantoTM II flow cytometer (10^4 cells captured per condition), and analyzed with BD FACSDiva software. The percentages of FLICA-positive cells were expressed as mean ± SEM of three replicates.

Immunofluorescence

AIF expression and cellular localization was assessed by immunofluorescence. Briefly, osteoblasts were plated on coverslips (2.4 x 10^5 cells/12-well), treated with the indicated reagents, fixed, permeabilized, and incubated with antibodies targeting AIF (1:25) overnight at 4°C after blocking non-specific antibody binding with 10% FBC (Autobiochlear)/ 1% Triton X-100 for 1 h. Then, cells were incubated with Alexa Fluor 555 goat anti-rabbit IgG (1:1000) for 1 h and mounted with DAPI staining. Expression and cellular localization of the antigen were visualized with a Leica TCS SP5 confocal microscope (Mannheim, Germany).

Statistical analysis

All data are presented as means ± SD from 3 to 7 replicates. Student's *t*-test or ANOVA with Bonferonni correction were utilized to analyze differences between treated samples and controls. Statistical significance was defined as **P<.05*, ***P<.01*, and ****P<.001*. Experiments were repeated for verification at least three times. Study duration was from September 2007 to December 2020.

3. RESULTS

Exogenous ceramide reduces osteoblast viability

Ceramide effect on osteoblast viability was initially evaluated with the MTT assay by culturing MC3T3-E1, SaOS-2, and POB cells with C_2-ceramide, a short chain ceramide analogue that mimics the biological effects of endogenous ceramide (Cuvillier et al., 1996), for 24 hours. Osteoblasts exposed to increasing concentrations of C_2-ceramide demonstrated a dose-dependent decrease in viability (Figure 1A). Although POB cells were more sensitive to 10 and 20 μM C_2-ceramide, demonstrating a 33% reduction in cell viability at these concentrations, all three osteoblast cell types demonstrated a 40-70% reduction in cell viability with 50-150 μM C_2-ceramide (Figure 1A). To evaluate the time-dependent effects of ceramide on osteoblasts, MC3T3-E1 cells were treated with 100 μM C_2-ceramide at various time points. Figure 1B shows that C_2-ceramide induced a time-dependent reduction in osteoblast viability, with a 31.6% reduction in cell survival at 12 hours and 94.6% at 48 hours. To confirm that the changes in cell viability were due to the effect of C_2-ceramide, MC3T3-E1 cells were treated with the inactive ceramide analogue, dihydroceramide (C_2-DHC), for 24 hours, and effects on viability were assessed with the MTT assay. Exogenous C_2-DHC had no effect on osteoblast viability (Figure 1C), confirming the specificity of the active C_2-ceramide compound in decreasing viability. Finally, phase-contrast microscopy of the cells exposed to C_2-ceramide showed morphological changes typical of apoptosis, specifically cell shrinkage and membrane blebbing (Figure 1D).

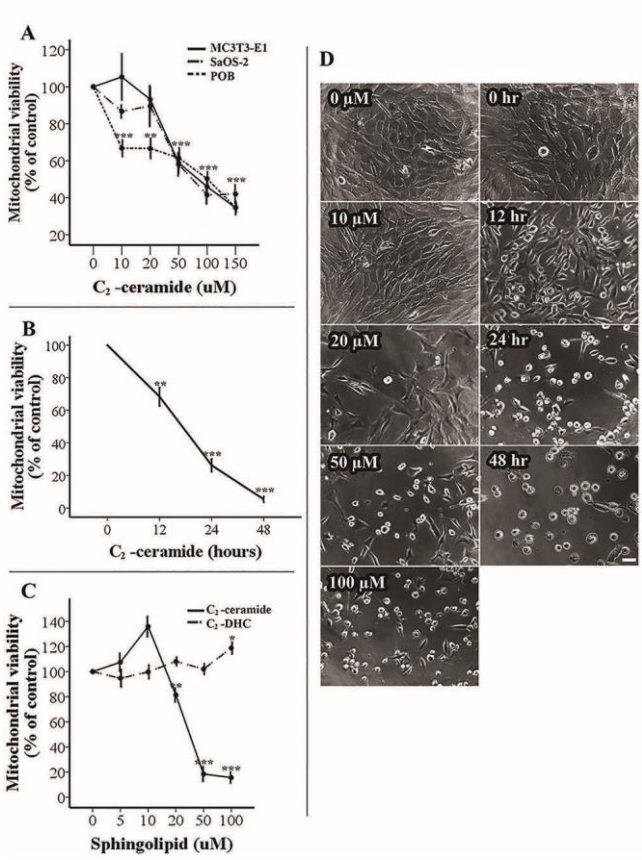


Figure 1 C_2-ceramide dose- and time-dependently reduces MC3T3-E1 cell viability and dose-dependently reduces SaOS-2 and POB cell viability. (A) MC3T3-E1, SaOS-2, and POB cells were treated with C_2-ceramide for 24 h at the indicated concentrations, after which viability was measured with the MTT assay. C_2-ceramide significantly reduced viability in a dose-dependent manner in all cell lines. Bonferroni testing shows a significant difference from control at $^{**}P < 0.01$ and $^{***}P < 0.001$. (B) MC3T3-E1 cells were treated with 100 μM C_2-ceramide for the indicated time periods before viability was measured. C_2-ceramide time-dependently reduced osteoblast viability ($^{***}P < 0.001$). Bonferroni testing shows a significant difference from respective controls ($^{**}P < 0.01$ and $^{***}P < 0.001$). (C) MC3T3-E1 cells were treated with100 μM C_2-ceramide or C_2-DHC for the indicated time periods before viability was measured. C_2-DHC did not reduce osteoblast viability at the tested concentrations. Bonferroni testing shows a significant difference from respective controls ($^{*}P < 0.05$; $^{**}P < 0.01$; ***and $P < 0.001$. (D) MC3T3-E1 cells were treated with C_2-ceramide (left panel) for 24 h at the indicated concentrations and C_2-ceramide (100 μM) treatments (right panel) for the indicated incubation periods. Cell morphology was assessed by phase contrast microscopy. Images were taken from representative fields. Scale bar is 100 μm in length.

Exogenous ceramide is a slow inducer of apoptosis in osteoblasts

We next examined whether cells exposed to C_2-ceramide exhibited any of the other feature's characteristic of apoptosis. MC3T3-E1 cells treated with C_2-ceramide for 24 and 48 hours exhibited a dose-dependent increase in the percentage of PI uptake, with 100 μM C_2-ceramide producing an increase in PI uptake and apoptosis of over 80% at both time points (Figure 2A). We next assessed DNA fragmentation by labelling double-stranded DNA breaks using the TUNEL assay. MC3T3-E1 cells cultured in the absence of C_2-ceramide for 24 hours demonstrated no DNA fragmentation (Control). However, TUNEL staining of osteoblasts cultured with C_2-ceramide for 24 hours confirmed that apoptosis had taken place, consistent with the MTT assay and PI staining results (Figure 2B).

To study the type of cell death induced by C_2-ceramide, the nuclear morphology of the osteoblasts was examined. When MC3T3-E1 cells were treated with C_2-ceramide for 48 hours and their nuclei stained with Hoechst 33324, fluorescence microscopy revealed that there was chromatin condensation characteristic of apoptosis and resembling the nuclear morphology of cells treated with staurosporine (SS), a potent apoptosis inducer; however, C_2-ceramide was a slower inducer of apoptosis than SS (Figure 2C). This effect was specific to C_2-ceramide, as C_2-DHC treated cells showed no morphological changes compared to untreated controls (Figure 2C).

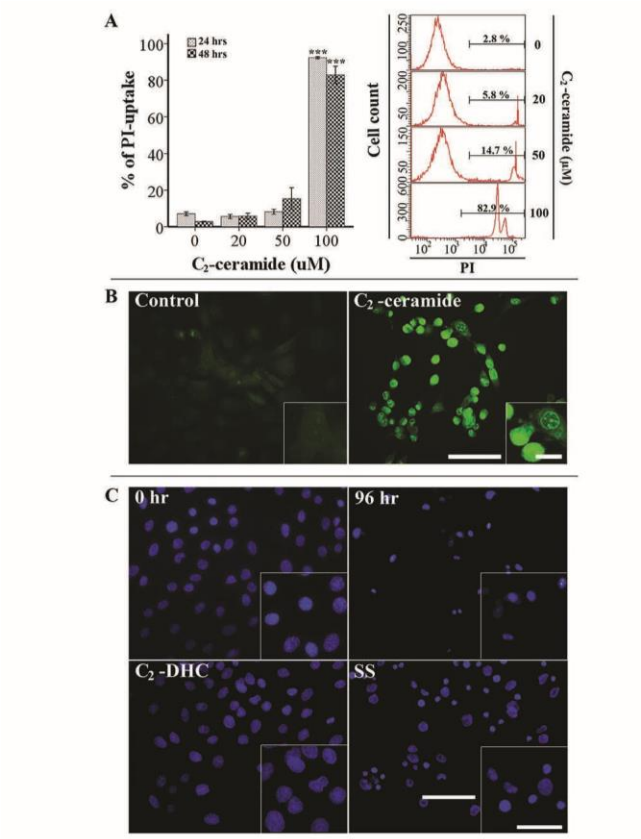


Figure 2 C_2-ceramide is a slow inducer of apoptotic cell death in osteoblasts. (A) MC3T3-E1 cells were treated with C_2-ceramide at the indicated concentrations for 24 and 48 h, then cells were labelled with PI. PI uptake was measured by flow cytometry. (A, left panel) C_2-ceramide dose-dependently increased the percentage of PI uptake at both time points. These points were significantly different from respective controls (***$P<$ 0.001). (A, right panel) Representative histograms from PI-stained samples at 48 h. (B) MC3T3-E1 cells were treated with vehicle or C_2-ceramide (100 μM) for 24, then cells were labelled with TUNEL and visualized by fluorescence microscopy. Images are taken from representative fields. Scale bars are 50 and 12.5 μm in length. (C) C_2-ceramide (100 μM) C_2-DHC (100 μM) and SS (10 μM) for the indicated time points. Cells were stained with Hoechst 33324 and visualized by fluorescence microscopy. Images are taken from representative fields. Scale bars are 50 and 12.5 μm in length.

Exogenous ceramide induces caspase-8-dependent osteoblast cell death

It is established that both the initiator capsases-2, -8, -9, and -10 and the executioner caspases-3, -6, and -7 play vital roles in cellular functions (Shalini et al., 2015). To determine whether ceramide activates caspases, MC3T3-E1 cells were pre-treated with the cell-permeable pan-caspase inhibitor DEVD-CHO, which inhibits caspases-3, -6, -7, -8, and 10; mitochondrial viability was then measured using the MTT assay. DEVD-CHO partially rescued the C_2-ceramide-induced reduction in osteoblast viability by 26.5%

during a 24-hour culture period (Figure 3A). The effects of C_2-ceramide on pan-caspase activity were assessed by staining with FLICA, which binds covalently with active caspases to retain a fluorescent signal. Flow cytometry analysis showed that C_2-ceramide significantly increased FLICA-positive cells by 17.7% compared to controls (Figure 3B). Thus, caspases appear to be mediators of C_2-ceramide-induced cell death in osteoblasts.

To elucidate which specific caspases are activated by ceramide, MC3T3-E1 cells were pre-treated with specific caspase-8 and -9 inhibitors and their effects on C_2-ceramide-mediated osteoblast viability assessed. The caspase-8 inhibitor significantly rescued the C_2-ceramide-induced reduction in osteoblast viability by 28% (Figure 3C). In contrast, the caspase-9 inhibitor resulted in a non-significant reversal in osteoblast viability by 12.1% (Figure 3C). We then investigated the effects of C_2-ceramide on caspase-3 and -7 activities. Western blot analysis of analysis of caspase-3, -7 and *poly ADP-ribose polymerase* (PARP) demonstrated that C_2-ceramide failed to actively cleave these proteins. However, SS, a known caspase activator in Jurkat T cells, induced active cleavage in both Jurkat and MC3T3-E1 cells (Figure 3D). These results suggest that C_2-ceramide activates the initiator caspase 8 as a potential target in the signaling cascade of osteoblast cell death.

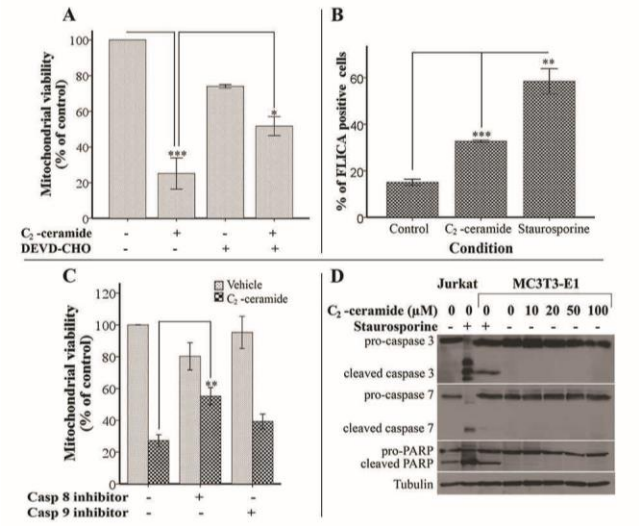


Figure 3 A specific caspase-8 inhibitor rescues C_2-ceramide-induced decreases in osteoblast viability. (A) MC3T3-E1 cells were pre-treated with 5 μM DEVD-CHO for 2 h and then C_2-ceramide (100 μM) for 24 h, and cell viability was measured with the MTT assay. DEV-CHO rescued the C_2-ceramide-induced reduction in osteoblast viability (*P< 0.05 and ***P< 0.001). (B) MC3T3-E1 cells were treated with vehicle, C_2-ceramide (50 μM) for 24 h, or SS (10 μM) for 3 h and then cells were labelled with FLICA and analyzed by flow cytometry. C_2-ceramide significantly increased the proportion of FLICA-positive cells (**P< 0.01 and ***P< 0.001). (C) MC3T3-E1 cells were pre-treated with specific caspase-8 and -9 inhibitors for 2 h and then C_2-ceramide (50 μM) for 24 h. Cell viability was measured with the MTT assay. The caspase-8 inhibitor rescued the C_2-ceramide-induced reduction in viability (**P< 0.01) but the caspase-9 inhibitor did not (P> 0.05). (D) MC3T3-E1 cells were treated with C_2-ceramide at the indicated concentrations for 5 h and SS (10 μM) for 3 h. As positive controls, Jurkat cells were treated with vehicle and SS (10 μM) for 3 h and the effects were assessed by western blotting analysis of caspase-3, -7 and PARP cleavage.

Exogenous ceramide induces p38-dependent osteoblast apoptosis

As ceramide was reported to modulate stress signaling cascades such as MAPK (Turjanski et al., 2007), we next investigated the effects of ceramide on activation of MAPK pathway members in MC3T3-E1 cells. C_2-ceramide induced a dose-dependent increase in p38 and ERK phosphorylation; however, it had no effect on JNK phosphorylation (Figure 4A). Furthermore, C_2-ceramide induced p38 and ERK phosphorylation at early time points, with an activation peak at 5 hours and 10 minutes, respectively (Figure 4B). To investigate whether this C_2-ceramide-induced p38 and ERK activation but not JNK activation also occurred in primary murine osteoblasts, POB cells were treated for 5 hours with different C_2-ceramide concentrations. Indeed, C_2-ceramide induced dose-dependent phosphorylation of both p38 and ERK but not JNK, as assessed by western blotting (Figure 4C).

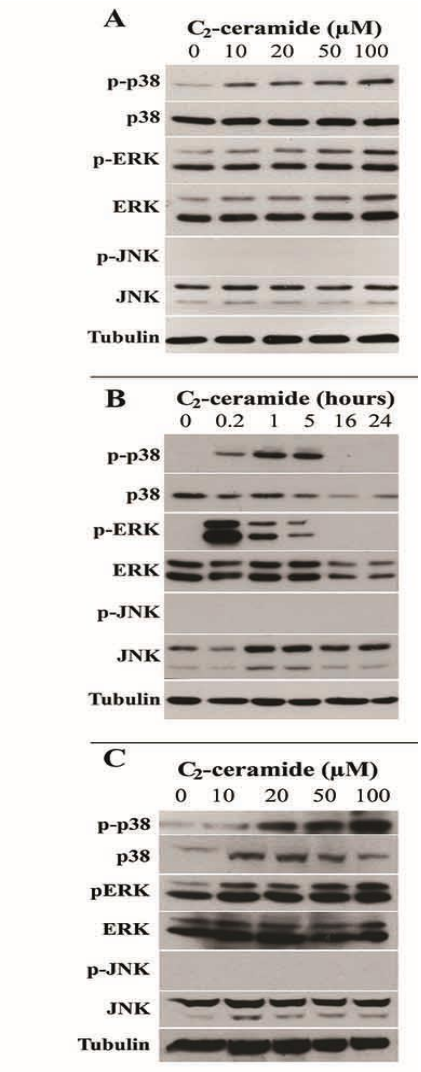


Figure 4 C_2-ceramide dose- and time-dependently activates p38 and ERK but not JNK in MC3T3-E1 and POB cells. (A) MC3T3-E1 cells were treated with C_2-ceramide for 5 h at the indicated concentrations or (B) with C_2-ceramide (100 μM) for the indicated time periods. (C) POB cells were treated with C_2-ceramide for 5 h at the indicated concentrations. Effects on MAPK protein phosphorylation was assessed by western blotting.

We then investigated the role of p38 and ERK activation on C_2-ceramide-mediated osteoblast apoptosis using two inhibitors: SB202190 (an inhibitor of p38 phosphorylation) and PD98059 (an inhibitor of ERK phosphorylation). Western blot analysis showed that both inhibitors partially reduced C_2-ceramide-induced p38 and ERK phosphorylation (Figure 5A, B, upper panels). The effects of these inhibitors on C_2-ceramide-induced apoptosis were further studied using PI uptake and flow cytometry. Only SB202190 significantly rescued the C_2-ceramide effect, as shown by an 8.5% increase in PI uptake (Figure 5A, lower panel). PD98059 had only a minor effect in reducing the C_2-ceramide effect, as shown by a 5.5% increase in PI uptake (Figure 5B, lower panel). These results suggest that C_2-ceramide-induced p38 activation is a downstream result of C_2-ceramide-induced loss of cytoplasmic integrity as part of the PCD pathway in osteoblasts.

Finally, to assess the sequence of ceramide-induced caspase-8, p38, and ERK activation in inducing osteoblast PCD, MC3T3-E1 cells were treated with a caspase-8 inhibitor and the effects on C_2-ceramide-induced p38 and ERK activation were assessed by western blotting and densitometry. The caspase-8 inhibitor partially rescued C_2-ceramide-induced p38 but not ERK phosphorylation (Figure 5C). Thus, ceramide-induced caspase-8 activation appears to be upstream of p38 phosphorylation, whereas C_2-ceramide-induced ERK activation does not appear to be a part of the C_2-ceramide-induced PCD pathway in osteoblasts.

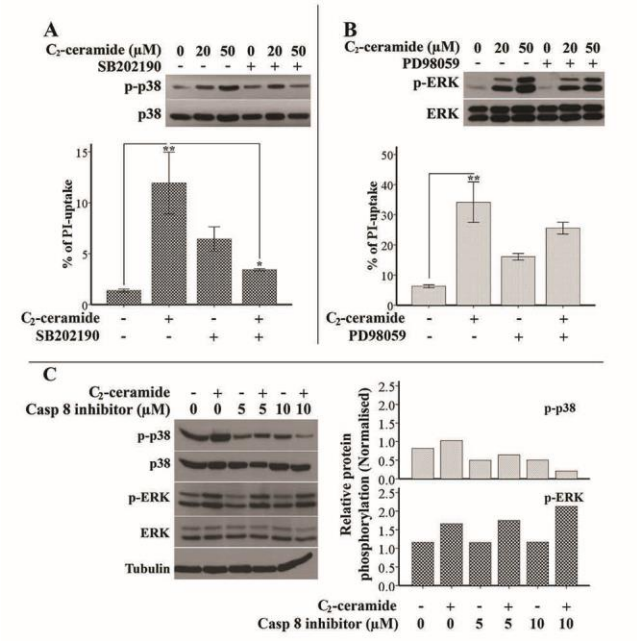


Figure 5 C_2-ceramide induces p38-dependent and ERK-independent increases in cytoplasmic permeabilization, and C_2-ceramide-induced caspase-8 activation is upstream of C_2-ceramide-induced p38 activation but not ERK activation. (A) MC3T3-E1 cells were pre-treated with SB 202190 (10 μM) or (B) PD 98059 (20 μM) for 2 h and then 20 or 50 μM C_2-ceramide for 1 h or 20 μM of C_2-ceramide for 24 h. The effects of inhibitors on C_2-ceramide-induced p38 and ERK phosphorylation were assessed by western blotting (upper panels), and C_2-ceramide-induced cell death was assessed by PI staining and flow cytometry (lower panels). SB 202190 but not PD 98059 rescued the C_2-ceramide-induced cell death in osteoblasts (**P< 0.01 and P> 0.05, respectively). (C) MC3T3-E1 cells were pre-treated with caspase-8 inhibitor at the indicated concentrations for 2 h and then 20 μM C_2-ceramide for 1 h. Effects on p38 and ERK phosphorylation were assessed by western blotting and densitometry.

4. DISCUSSION

Programmed cell death of osteoblasts is an important parameter in skeletal homeostasis that, when dysregulated, may contribute to metabolic bone diseases (Weinstein and Manolagas, 2000; Wilson and Kumar, 2018). Osteoblasts are believed to undergo apoptosis under the influence of hormones and cytokines that regulate their integrity (Komori, 2016). Therefore, identifying compounds that influence this process and elucidating the mechanisms by which these compounds exert their effects will be useful for understanding osteoblast-induced bone pathology and designing targeted interventions. Ceramide has emerged as an important second messenger that mediates cell death and plays important roles during cellular development (Hannun and Obeid, 2008). Furthermore, knockdown or mutation (*fro/fro* mouse) of the gene encoding nSMase2 (neutral sphingomyelinase, an enzyme involved in ceramide synthesis) results in mice with short stature or bone fragility (Aubin et al., 2005; Stoffel et al., 2005; Stoffel et al., 2007). This suggests an important physiological role for this enzyme, specifically ceramide, in bone development and/or remodeling. However, the molecular events underpinning ceramide-induced cell death in osteoblasts have not been fully elucidated.

C_2-ceramide, a short-chain analogue of ceramide, has been used to study ceramide-induced cell death in various cell types because it was shown to mimic the biological effects of endogenous ceramide (Guenther et al., 2008; Zhu et al., 2014). Here we show that physiologically relevant concentrations of C_2-ceramide caused the following effects in osteoblasts *in vitro:* (1) promoted apoptosis in a dose- and time-dependent manner; (2) apoptosis involved the activation and activity of MAPK, p38, but not JNK or ERK1/2; (3) the initiator caspase-8 activated p38 and (4) the executioner caspases 3, 7, and 9 and the downstream mediator of cell death PARP were not involved in osteoblast apoptosis.

Our demonstration that p38 MAPK is activated by ceramide and that its specific inhibitor SB202190 partially abrogated the effect of ceramide on osteoblast apoptosis indicates that the increased activity of this MAPK might be one way in which ceramide causes PCD in osteoblasts. It is known that p38 and JNK play important roles in modulating apoptosis caused by various stimuli (Chang and Karin, 2001; Zarubin and Han, 2005), and they increase NF-kB activity in vascular endothelial cells (Ho et al., 2006). Other has shown that sphingomyelinase is involved in osteoblasts apoptosis via p38 MPA kinase (Kozawa et al., 1999). In addition,

ceramide was found to act as second messenger in TNF-α induced apoptosis in osteoblasts. C2-ceramide activated NF-kB (Chae et al., 2000; Kitajima et al., 1996). Interestingly, it seams that C2-ceramide and sphingomyelinase may induce cell dealt or survival depending on cellular concentration (Hill and Tumber, 2010). Although JNK and ERK1/2 can modulate apoptosis caused by various stimuli in other systems (Dhanasekaran and Reddy, 2008; Jeon et al., 2007), our data suggest that they are not involved in ceramide-induced apoptosis in mouse osteoblasts.

The activation of p38 in ceramide-induced cell death has been reported to be upstream (Bao and Shi, 2007; Kim et al., 2008) or at the level of mitochondria (Bao and Shi, 2007; Kong et al., 2005). In this report, C_2-ceramide induced p38 phosphorylation after 10 minutes of treatment, and the caspase 8 inhibitor rescued C_2-ceramide-induced p38 phosphorylation. Accordingly, this suggests that p38 activation is an early event, most probably upstream of mitochondria yet downstream of caspase 8 activation in the C_2-ceramide-induced cell death signaling pathway in osteoblasts. In other words, C_2-ceramide possibly induces cell death through the extrinsic apoptotic pathway by binding to death receptors and activating caspase 8. Caspase 8 subsequently activates a cascade of events, including p38 activation that will lead to apoptotic cell death (Yang et al., 2017).

PCD is initiated by two major caspase cascades. The first is initiated by the activation of cell surface death receptors, such as the TNFα receptor and Fas, activating the initiator caspase-8, which in turn cleaves the downstream executioner caspases-3, -6, and -7 (Lavrik et al., 2005). The second is triggered by the release of mitochondrial cytochrome c, which promotes the formation of the multi-subunit complex Apaf-1 and pro-caspase-9, leading to the execution of apoptosis, mainly by caspase-3 (Green, 2005). Since caspase inhibition and p38 inhibition only minimally protected osteoblasts against ceramide, it is likely that compensatory osteoblast cell deathpathways exist. In certain osteoblast death paradigms, caspase inhibition may result in the activation of compensatory cell death processes. Various reports have demonstrated that ceramide could either induce caspase-dependent cell death or caspase-independent cell death in response to the intrinsic apoptotic cascade (Heinrich et al., 2004; Rotolo et al., 2005).

In addition, to the C_2-ceramide activated extrinsic apoptotic pathway (caspase-8/p38 induced cell death) we demonstrate (initial results) that C_2-ceramide may induce the release of mitochondrial intermembrane space proteins, which may act as mediators of C_2-ceramide-induced programmed death signaling pathways in osteoblasts (Supplementary Figure 1). This may indicate that ceramide utilizes more than one signal transduction pathway at different sub-cellular locations to ultimately cause cell death in osteoblasts. Alternatively, these molecular activations may represent a single pathway whereby ceramide-induced caspase 8 and p38 activation maylead to MOMP and the ultimate release of pro-apoptotic proteins and cell death. Taken together, these results allow the formulation of a working model of C_2-ceramide-induced cell death signaling pathway in osteoblasts (Figure 6).

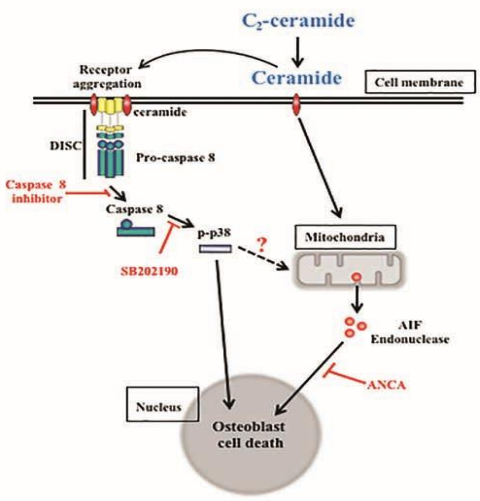


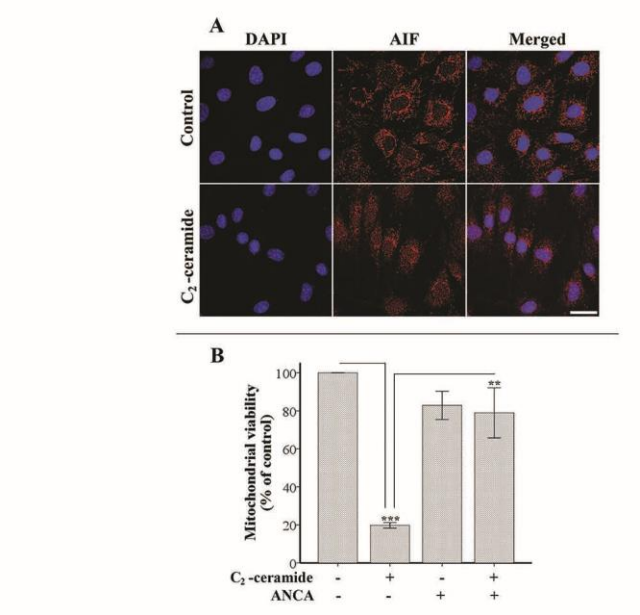
Figure 6 A working model of ceramide-induced cell death signaling in osteoblasts.

Ceramide possibly initiates its death inducing signaling at the cytoplasmic membrane, where it facilitates death receptor aggregation, which in turn promotes caspase-8 autophosphorylation. Subsequently, induction of p38 phosphorylation occurs upstream of the mitochondria. SB202190; is a potent cell permeable inhibitor of p38 MAPK and ANCA; a potent inhibitor of protein-nucleic acid interactions.

5. CONCLUSION

All metabolic bone diseases show an imbalance in bone remodeling. This imbalance could partly be due to disturbances in osteoblast PCD. Here we highlighted a novel role for ceramide in inducing caspase-8 and p38-dependent osteoblast PCD. Ceramides are important second messengers that mediate cell death and play an important role during bone development. Thus, understanding ceramide-induced cell death molecular pathways in osteoblasts could help in the development of new targeted therapies that can selectively identify cells with altered PCD, not only to treat metabolic bone diseases but also to treat other devastating diseases such as head and neck cancer.

Supplementary Data



Supplementary Figure 1 C_2-ceramide induces AIF nuclear translocation and ANCA rescues C_2-ceramide-induced reductions in osteoblast viability. (A) MC3T3-E1 cells were treated with or without C_2-ceramide (50 μM) for 24 h. Effects of C_2-ceramide on the localization of AIF were assessed by immunostaining and visualized by confocal microscopy. Images are from representative fields. Scale bar is 50 μm in length. (B) Pre-treated with ANCA (100 μM) for 2 h and then C_2-ceramide (100 μM) for 24 h. Cell viability was measured with the MTT assay. ANCA rescued the C_2-ceramide-induced reduction in osteoblast viability (**P< 0.01 and ***P< 0.001).

Authors' contributions

This work was carried out in collaboration among all authors.

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Conflict of interest

The authors declare that there are no conflicts of interests.

Ethical approval

All work was performed at King's College London in accordance with UK Home Office Project License P8D5E2773 (K.J.L.).

Data and materials availability

All data associated with this study are present in the paper.

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